Sequence and Expression Patterns of Mouse SPR1: Correlation of Expression with Epithelial Function

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A final event in the terminal differentiation of stratified squamous epithelia is the formation of a cornified cell envelope, which is a complex of several proteins cross-linked together by transglutaminases. One set of proteins is the family of small proline rich (SPR) proteins. In human foreskin epidermal cell envelopes, SPRs serve as cross-bridging proteins among the more abundant loricrin. In order to study further their evolution and expression, we have isolated and sequenced cDNAs encoding two mouse SPR1 proteins, SPR1a and SPR1b. Comparative sequence analyses showed the preservation of the overall structure of mammalian SPR1 proteins with highly conserved termini and a central peptide domain repeated 13 (SPR1a) or seven (SPR1b) times. Tissues obtained from mouse fetal, newborn, and adult skin were tested by Northern blot analyses, *in situ* hybridization, and immunohistochemistry using an antibody raised to a synthetic peptide corresponding to the C terminus of the SPR1a protein. Skin expression was first detected in fetal periderm, in anagen hair follicles of newborn and older mice, and in the thickened epidermis of the lip and footpad, but no signal was detected in interfollicular trunk epidermis. High levels of SPR1a expression were found in epithelia from the forestomach and penis, and in benign squamous papillomas. Other epithelia expressing SPR1a include the tongue, esophagus, and vagina. Whenever detected, SPR1a positive staining was present in the spinous and granular layers. In the forestomach and papillomas, the periphery of cells in the cornified layer was also stained. Our results suggest that SPR1a participates widely in the construction of cell envelopes in cornifying epithelia characterized by either increased thickness or a requirement for extreme flexibility. Based on its likely function as a cross-bridging protein in cell envelopes, we conclude that the mechanical attributes of cell envelopes may be determined in part by the SPR1 content, in accordance with the specific function of the epithelium. *Key words: small proline rich/cDNA/epithelium. J Invest Dermatol 106:294–304, 1996*

Terminal differentiation in the epidermis is a multistage process during which keratinocytes, the major cell type, cease proliferation, migrate from the proliferative basal layer, express new sets of structural proteins and enzymes, eventually lose nuclei, change their shape to flattened squames, and die. The cornified keratinocyte consists largely of aggregated keratin filaments embedded in an interfilamentous matrix of filaggrin, enclosed within a highly insoluble cornified cell envelope (CE). Originally, it was thought that the CE is the result of a random cross-linking of cellular proteins by activated transglutaminases [30,35], however, recent advances in the identification of CE structural proteins, and biochemical analyses of the CE composition, have revealed a highly ordered and complex structure [11,34,43–45]. Proposed CE proteins include involucrin [9–11,36,54,55], loricrin [5,20,29,56], keratolinin/cystatin α [58,46], SKALP/elafin [31,32,44], trichohyalin [25], and a new family of proteins, termed small proline rich proteins (SPR) [4,13,23,24], which includes cornifin [12,27,42] and pancornulins [16,33]. These proteins are thought to be cross-linked together by both disulfide bonds and the N^{ϵ}-(γ-glutamyl)lysine isodipeptide bond formed by the action of transglutaminases [20,40,41,44,48].

Many questions concerning the expression, structure, and role of these protein components in the CE remain unresolved. For example, the expression of SPRs is complex. They consist of at least three closely related subfamilies, SPR1, SPR2, and SPR3 [13]. They are rich in proline (up to 40%) and contain internal repeating peptide units of 8 (SPR1 and SPR3) or 9 (SPR2) amino acids. Recently, rabbit (cornifin [27]), pig [47], and monkey [1] homologs of the human SPR1 gene have been cloned. SPRs were originally isolated by differential screening as ultraviolet-inducible genes [22,24] from human keratinocyte cDNA libraries. Subsequently, expression of SPR genes was shown to be modulated during

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Abbreviations: SPR, small proline rich; CE, cornified envelope.

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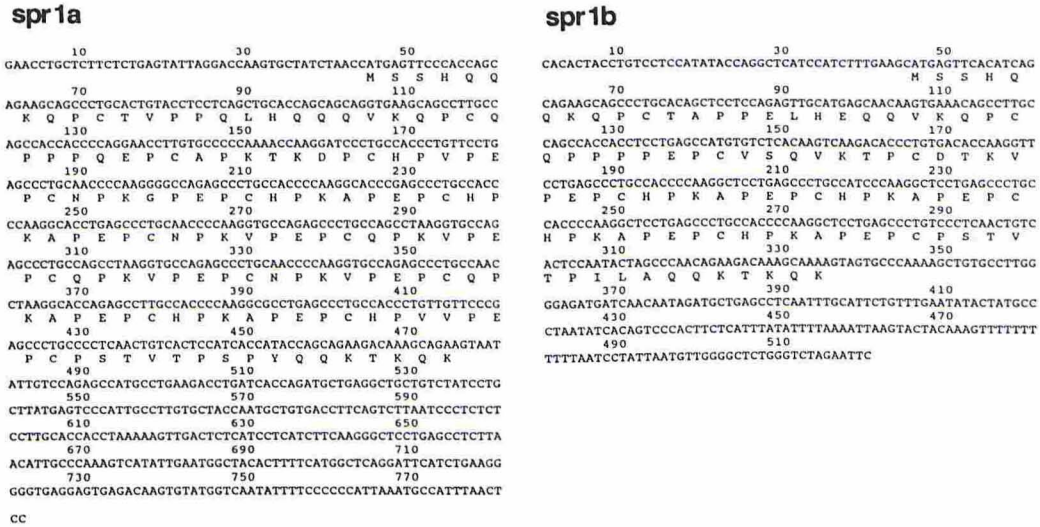


Figure 1. Complete nucleotide sequence of SPR1a (*left*) and SPR1b (*right*) cDNAs. Open reading frame is indicated by the one letter code. Accession numbers are X91824 and X91825.

keratinocyte differentiation [14,23], senescence [15,39], and by differentiation-inducing agents such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [3,14,24,53], retinoids [2,12,27,47], cyclic AMP [3], and interferon-γ [38]. Recently, it was demonstrated that the expression of one of the SPR genes, SPR1, is induced in skin after ultraviolet irradiation [52]. Human genes encoding SPR1, SPR2, and SPR3 were mapped as a cluster on chromosome 1q21.1, in close proximity with two other CE precursors, involucrin and loricrin, as well as two other epidermal differentiation-specific genes, profilaggrin and trichohyalin [13,49]. The genomic organization of all these genes, as well as the overall structure of their proteins, is similar in certain ways. The genomic sequences contain only one intron in the 5′-noncoding region. In the coding region, the bulk of the sequences consist of the multiple tandem peptide repeats, the sequences of which differ markedly among the various proteins. This has been taken to suggest not only their common evolutionary origin, but also similar expression and function in the terminal differentiation program of stratified squamous epithelia [4].

Recently, it was demonstrated that treatment of cultured rabbit tracheobronchial epithelial cells with a Ca^{2+}-ionophore induced the cross-linking of the rabbit SPR1 [27]. This suggested that SPRs may serve as substrates for transglutaminases and are perhaps precursors of the cornified cell envelope of stratified squamous epithelia. In concordance with this, pancornulins (human SPR1 and SPR3) have been shown to participate in the *in vitro* transglutaminase catalyzed reaction both as amino donor and amino acceptor [16]. Also, monomeric SPR proteins become more abundant in tissues exposed to transglutaminase inhibitors [21]. Furthermore, recent analyses of the amino acid compositions of human foreskin and newborn mouse epidermal CEs indicated that SPRs might comprise up to 5% of the mature CEs [43,45]. Indeed, recent microsequencing of peptides derived from human foreskin epidermal CEs have now provided the ultimate proof that SPR1 and SPR2 proteins are isodipeptide cross-linked components [44]. In the outer portion of this CE structure, SPR1 and SPR2 constituted ~2–3% each of the molar mass of the CE protein, whereas loricrin constituted ~95%. Thus, the SPRs appear to function as cross-bridging proteins by cross-linking to loricrin by way of certain glutamines and lysines on their terminal (but not central repeating) domain sequences [44]. This pattern raises the interesting question of the precise function of the SPRs in the CE. One possibility is that mixtures of SPRs with loricrin and other CE proteins might alter the physical characteristics of the CE and thus the epithelium [44]; that is, the functions and properties of different epithelia may be influenced by both the level and type of SPR protein expressed. Accordingly, a detailed study of their expression in different epithelia is required. A first commendable study has shown widely varying expression in certain normal and pathological human epithelial tissues including the epidermis [21].

In the present work, we have studied the expression of SPR1 in many mouse stratified squamous epithelial tissues, and have identified a hitherto unrecognized correlation between SPR1 expression and epithelial tissue structure and function.

MATERIALS AND METHODS

Cells and Tissues Tissue samples were from CD-1, or Balb/C mice, except for penis, which was from nude mouse. Papillomas and carcinomas were obtained by a standard DMBA initiation and TPA promotion protocol [18,19].

Isolation of Mouse SPR cDNAs A CD-1 mouse papilloma cDNA library constructed in λgt10 was screened using human SPR1 (nucleotides 1–600 in clone 15B [24]) cDNA fragment as a probe. Hybridization and washing conditions were as described [26], except that the formamide concentration was lowered to achieve a 90% hybridization stringency. Nucleotide sequencing of both strands was determined by dideoxy sequencing [26] using specific primers and suitable internal restriction sites. For this, cDNAs were subcloned in pGEM3 vectors. In addition, sequences were determined directly from polymerase chain reaction fragments generated from λgt10 cDNA clones. For the SPR1 subfamily, 10 λgt10 cDNA clones were isolated and eight clones were sequenced. Two related sequences were identified and termed SPR1a and SPR1b.

Northern Blot Analyses Total RNA was isolated by the guanidine isothiocyanate/$CsCl_2$ protocol [26] from tissue samples (day 14 and 16 fetal CD-1 mouse skin, newborn and adult mouse skin, and mouse papillomas and carcinomas). Twenty micrograms of RNA were loaded per lane and separated by 1.5% agarose/formaldehyde gel electrophoresis, transferred to Hybond-N paper (Amersham, Tokyo, Japan) and hybridized at 42°C in 50% formamide/5 × SSPE [26] to the SPR1 probe labeled with ^{32}P dCTP by random priming (Promega, Tokyo, Japan). Hybridization to mouse α-tubulin served as a control.

***In Vitro* Synthesis of SPR1 Protein** Full-length SPR1a cDNA sequence **(Fig 1)** was cloned in the pGEM3 vector in two orientations. Sense and anti-sense SPR1a RNAs were transcribed from the DNA template by T7 RNA polymerase (Promega). The RNAs were extracted with phenol and were concentrated by ethanol precipitation. RNAs (1–4 μg) were translated in the *in vitro* reticulocyte lysate system (Promega). Proteins were labeled with ^{35}S-cysteine (Amersham), and separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were treated with Enlighting (Dupont, Biotechnology Systems, Tokyo, Japan), dried and exposed to Fuji x-ray film for 1–2 d.

Mouse SPR1a

N-terminus:
MSSHQQKQPCTVPPQLHQQQVKQPCQ
Repeat:

	1	2	3	4	5	6	7	8
1	P	P	P	Q	E	P	C	A
2	P	K	T	K	D	P	C	H
3	P	-	V	P	E	P	C	N
4	P	K	G	P	E	P	C	H
5	P	K	A	P	E	P	C	H
6	P	K	A	P	E	P	C	N
7	P	K	V	P	E	P	C	Q
8	P	K	V	P	E	P	C	Q
9	P	K	V	P	E	P	C	N
10	P	K	V	P	E	P	C	Q
11	P	K	A	P	E	P	C	H
12	P	K	A	P	E	P	C	H
13	P	V	V	P	E	P	C	P

C-terminus:
STVTPSPYQQKTKQK

Mouse SPR1b

N-terminus:
MSSHQQKQPCTAPPELHEQQVKQPCQ
Repeat:

	1	2	3	4	5	6	7	8
1	P	P	P	P	E	P	C	V
2	S	Q	V	K	T	P	C	D
3	T	K	V	P	E	P	C	H
4	P	K	A	P	E	P	C	H
5	P	K	A	P	E	P	C	H
6	P	K	A	P	E	P	C	H
7	P	K	A	P	E	P	C	P

C-terminus:
STVTPILAQQKTKQK

Pig SPR1

N-terminus:
MSSQQQKQPCTPPPQPQQQQVKQPCQ
Repeat:

	1	2	3	4	5	6	7	8
1	P	P	P	Q	E	P	C	V
2	P	K	T	K	E	P	C	H
3	P	K	V	P	E	P	C	Q
4	P	K	V	P	E	P	C	Q
5	P	K	V	P	E	P	C	H
6	P	K	V	P	E	P	C	Q
7	P	K	V	P	E	P	C	P

C-terminus:
SPVIPAPAQQKTKQK

Rabbit cornifin

N-terminus:
MSSQQQKQPCTLPPQLQQHQVKQPCQ
Repeat:

	1	2	3	4	5	6	7	8
1	P	P	P	Q	E	P	C	V
2	P	K	T	K	E	P	C	Q
3	P	K	V	P	E	P	C	Q
4	P	K	V	P	E	P	C	Q
5	P	K	V	P	E	P	C	Q
6	P	K	V	P	Q	P	C	Q
7	P	K	V	P	E	P	C	Q
8	P	K	V	P	E	P	C	Q
9	P	K	V	P	E	P	C	Q
10	P	K	V	P	E	P	C	Q
11	S	K	V	P	Q	P	C	Q
12	P	K	V	P	E	P	C	Q

C-terminus:
TKQK

Monkey SPR1

N-terminus:
MSSQQQKQPCTPPPQLQQQQVKQPCQ
Repeat:

	1	2	3	4	5	6	7	8
1	P	P	P	Q	E	P	C	I
2	P	K	T	K	E	P	C	L
3	P	K	V	P	E	P	C	H
4	P	K	V	P	E	P	C	Q
5	P	K	V	P	E	P	C	H
6	P	K	V	P	E	P	C	P

C-terminus:
STVTPAPAQQKTKQK

Human SPR1

N-terminus:
MSSQQQKQPCIPPPQLQQQQVKQPCQ
Repeat:

	1	2	3	4	5	6	7	8
1	P	P	P	Q	E	P	C	I
2	P	K	T	K	E	P	C	H
3	P	K	V	P	E	P	C	H
4	P	K	V	P	E	P	C	Q
5	P	K	V	P	E	P	C	H
6	P	K	V	P	E	P	C	P

C-terminus:
SIVTPAPAQQKTKQK

Figure 2. Structural organization of the SPR1 proteins from several species: conservation of termini but variation in numbers of repeating elements.

Generation and Characterization of SPR1a Antibody A peptide was synthesized corresponding to the C terminus of the SPR1a protein. SPR1a C-terminal peptide: (C)PCPSTVTPSPYQQKTKQK. Cysteine (C in parenthesis) was added for more efficient coupling. Peptide was bound to bovine serum albumin (BSA) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester [17]. Specific antibody was generated in rabbits and named SPR1-Cterm. Antibody was purified using affinity chromatography [50] to peptide covalently bound to Sepharose 4B (Pharmacia, Tokyo, Japan). The antibody titer (1/500) and specificity were determined by enzyme-linked immunosorbent assay [17], as well as by Western blotting of *in vitro* synthesized SPR1a **(Fig 3)** and SPR2 (Kartasova *et al*, unpublished observations) proteins.

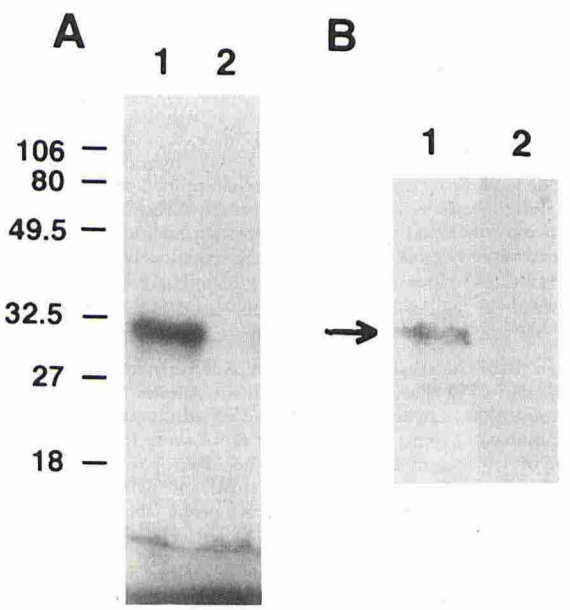


Figure 3. *In vitro* synthesized SPR1a protein is recognized by an antibody generated against the C-terminal peptide. *A*) RNAs were translated in the *in vitro* reticulocyte lysate system in the presence of ^{35}S-cysteine, and proteins were separated by 12.5% SDS-PAGE gel. *Lane 1*, sense SPR1a RNA; *lane 2*, anti-sense SPR1a RNA. Positions of molecular mass standards are indicated in kDa on the left. *B*) *Lane 1*, Recognition of the SPR1a protein by the SPR1-Cterm antibody; *lane 2*, blocking of the recognition by the SPR1-Cterm peptide.

Western Blotting Analyses Tissue samples were lyzed directly in buffer containing 2% SDS, 100 mM dithiothreitol, 60 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue and boiled for 5–10 min. Comparable amounts of proteins (except for papilloma extracts which have a very high content of SPR1, the amount loaded was one-fifth) were separated on 12.5% SDS-PAGE and blotted electrophoretically onto BA-S 83 nitrate membrane, pore size 0.2 μm (Schleicher and Schuell, Keene, NH) in Tris/Glycine buffer [17] at 100 V for 1 h. Migration to both positive and negative electrodes was analyzed by placing the membranes on both sides of the gel. In **Fig 3*B***, staining was done on the proteins transferred to the negative side, because of the high background from the reticulocyte lysate. After blotting, the membranes were incubated in Tris-buffered saline (TBS) buffer and blocked with 5% powdered milk in TBS. The SPR1-Cterm and K6 (rabbit polyclonal, Dr. S.H. Yuspa, NCI, Bethesda) antibodies were used at 1:500 dilution in 3% BSA in TBS. In control experiments, the SPR1-Cterm peptide was added at 0.1 mg/ml to inhibit the SPR1-Cterm antibody reaction. Bound antibody was detected using the ECL system (Dupont, Boston, MA) with horseradish peroxidase conjugated secondary antibody (Bio-Rad, Hercules, CA) at a 1:5000 dilution.

***In Situ* Hybridization** The *in situ* hybridization was performed as previously described [8]. The ^{35}S-labeled anti-sense and sense RNA probes were generated using full-length SPR1a cDNA in pGEM3 vector. Due to very high (85%) sequence homology, this probe probably cross-reacts with the SPR1b mRNA as well. Sense riboprobe was synthesized using SP6 RNA polymerase and a NdeI-linearized DNA template. Anti-sense riboprobe was synthesized using T7 RNA polymerase and a *Hin*dIII-linearized DNA template. Probes were used at 40×10^6 dpm/ml of hybridization buffer.

Immunohistochemistry Frozen sections were analyzed for the presence of SPR1 protein using affinity-purified SPR1a-Cterm antibody at

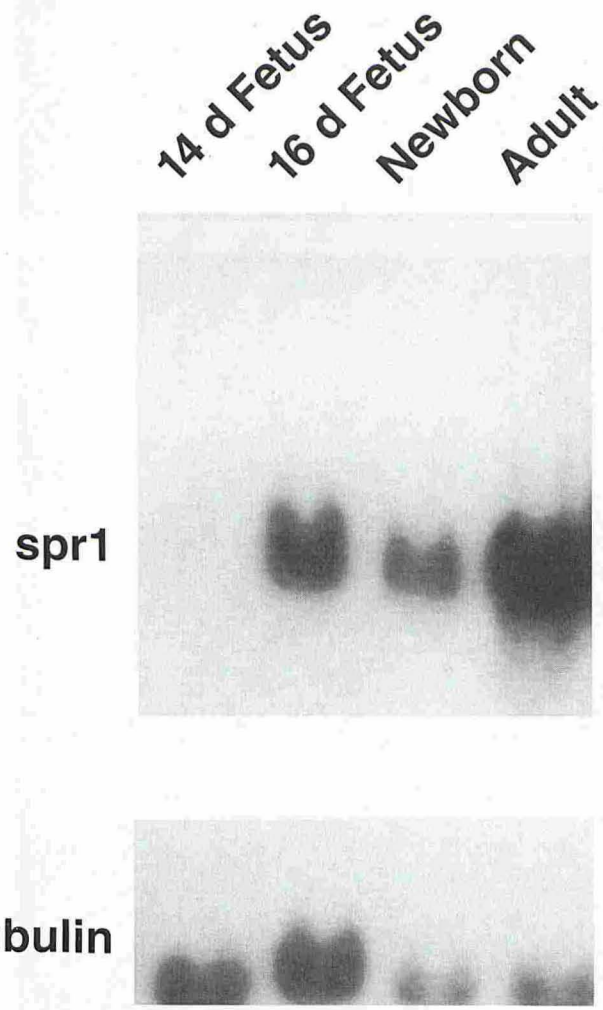


Figure 4. SPR1 mRNA is expressed in fetal periderm, hair follicles of newborn and adult mouse skin, and mouse papilloma as determined by Northern blot analyses. Total RNA (20 μg) was electrophoresed, transferred to Hybond-N paper, and hybridized to ^{32}P-labeled SPR1 probe. *Lane 1*, fetal skin day 14; *lane 2*, fetal skin day 16; *lane 3*, newborn skin; *lane 4*, adult skin. Control hybridization is to α-tubulin.

1:500 dilution in 10% BSA in PBS. Antibody binding was visualized by horseradish peroxidase conjugated second antibody (Kirkegaard and Perry Laboratory, Gaithersburg, MD).

RESULTS

A Mouse Papilloma cDNA Library Yields Two Distinct Sequences for the SPR1 Subfamily A mouse papilloma cDNA library constructed in λgt10 yielded two related SPR1 sequences: SPR1a, 782 nucleotides (five independent clones), and SPR1b, 520 nucleotides (three independent clones) (**Fig 1**, open reading frames are indicated). No homology was found between SPR1a and SPR1b in both the 5′- and 3′-noncoding regions. In the coding region, the overall homology between SPR1a and SPR1b is 85%. Thus, these cDNAs correspond to two independent SPR1 genes. The C+G content in the coding region is extremely high (i.e., 66–73%), while the noncoding regions are relatively A+T rich (i.e., 52–58%).

The mouse SPR1a (MW: 15,765) and SPR1b (MW: 10,609) proteins contain a central repeat of eight amino acids, flanked by 26 amino acids at the N-terminus and 15 amino acids at the C terminus (**Fig 1**). SPR1a and SPR1b proteins are extremely rich in proline (i.e., 32 and 27%, respectively). Glutamine residues (about 11%) are concentrated predominantly outside of the repeated domain. The length of the N and C termini is conserved with a few amino acid substitutions. In the central repeated domain, SPR1a and SPR1b contain different numbers of repeats: 13 and 7, respectively. Within the repeat, the sequence is well conserved except for the third and the eighth positions. At the third position, one of the following small hydrophobic amino acids is present: predominantly either valine or alanine, as well as glycine, proline (in the first repeat), or threonine (in the second repeat). At the eighth position, one of the following polar amino acids is present: predominantly either histidine or glutamine, as well as asparagine or aspartic acid; in the first and the last repeats, it is a hydrophobic amino acid. Thus, the consensus sequence for the central repeated unit of the mouse SPR1 proteins is: P - K - A/V - P - E - P - C - H/Q.

Comparison of the mouse SPR1 sequences (**Fig 2**) with the sequence of the SPR1 proteins from human [24], pig [47], monkey [1], and rabbit (cornifin [27]) reveals conservation in the length and sequence in the N and C termini (except for cornifin) of the SPR1 proteins and a variation in the number of central repeats. Mouse SPR1a and rabbit cornifin (SPR1) contain a net insertion of several more central repeats than mouse SPR1b or the other SPR1 proteins.

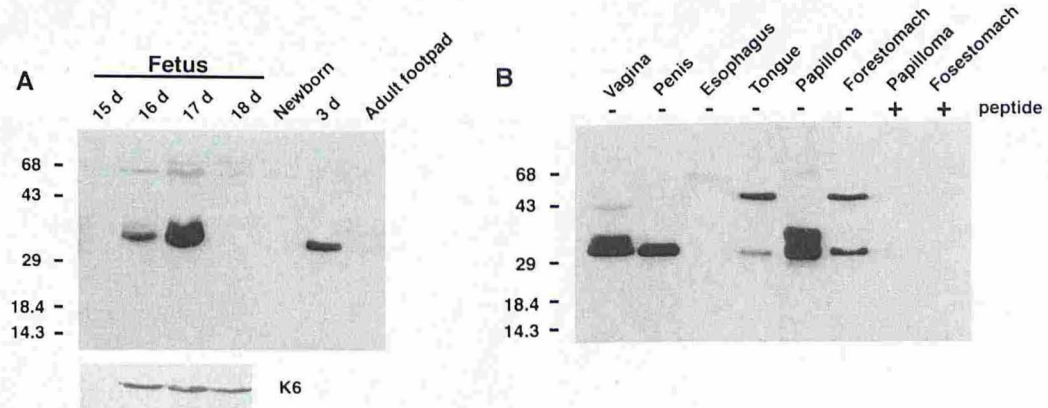


Figure 5. SPR1a protein is expressed in a variety of internal and external keratinizing epithelia as determined by Western blot analyses. Expression in mouse fetal, newborn, and adult skin (*A*); and in different epithelial tissues (*B*). Tissue samples were lyzed directly in a sample buffer (see *Materials and Methods*) and boiled for 5–10 min; comparable amounts of proteins (except for papilloma, where one-fifth was loaded) were separated by 12.5% SDS-PAGE, blotted onto BA-S 83 membrane, and probed with SPR1-Cterm antibody, which was used at 1/500 dilution in 3% BSA. Proteins were detected using ECL system. *A*) Samples from fetal (day 15–18), newborn, and 3-d-old skins, and from skin of adult foot pad; control staining with keratin 6 antibody of a parallel blot of the fetal skin samples is shown underneath. *B*) Samples from vagina, penis, esophagus, tongue, 22-wk-old papilloma induced by DMBA/TPA, and forestomach. Control blocking of the antibody reaction in the papilloma and forestomach samples were in the presence of the SPR1a-Cterm peptide. Positions of molecular mass standards are indicated in kDa on the left of each panel. Blocking of the reaction in papillomas and forestomach samples by the presence of the SPR1 peptide is shown in *Panel B*.

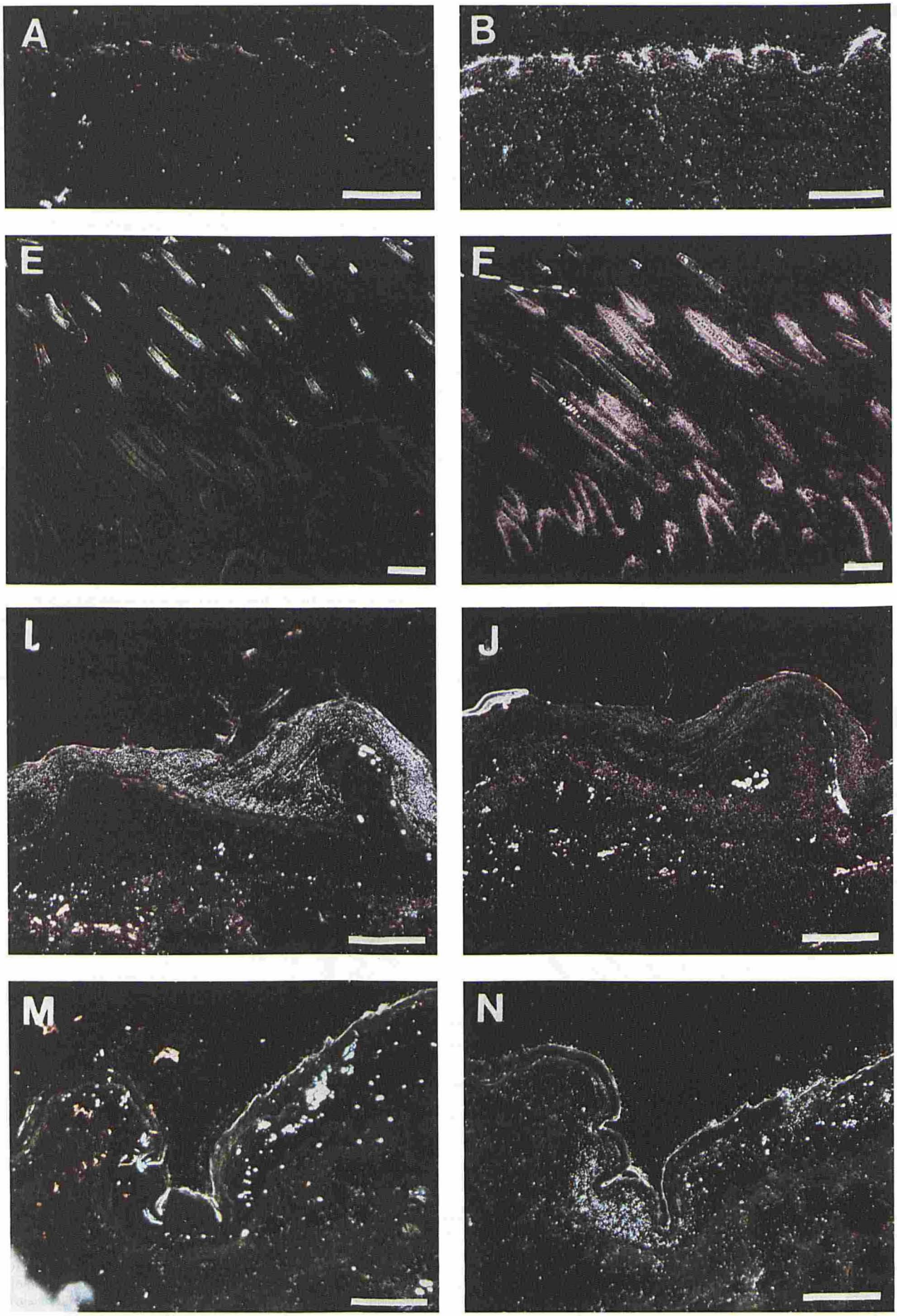
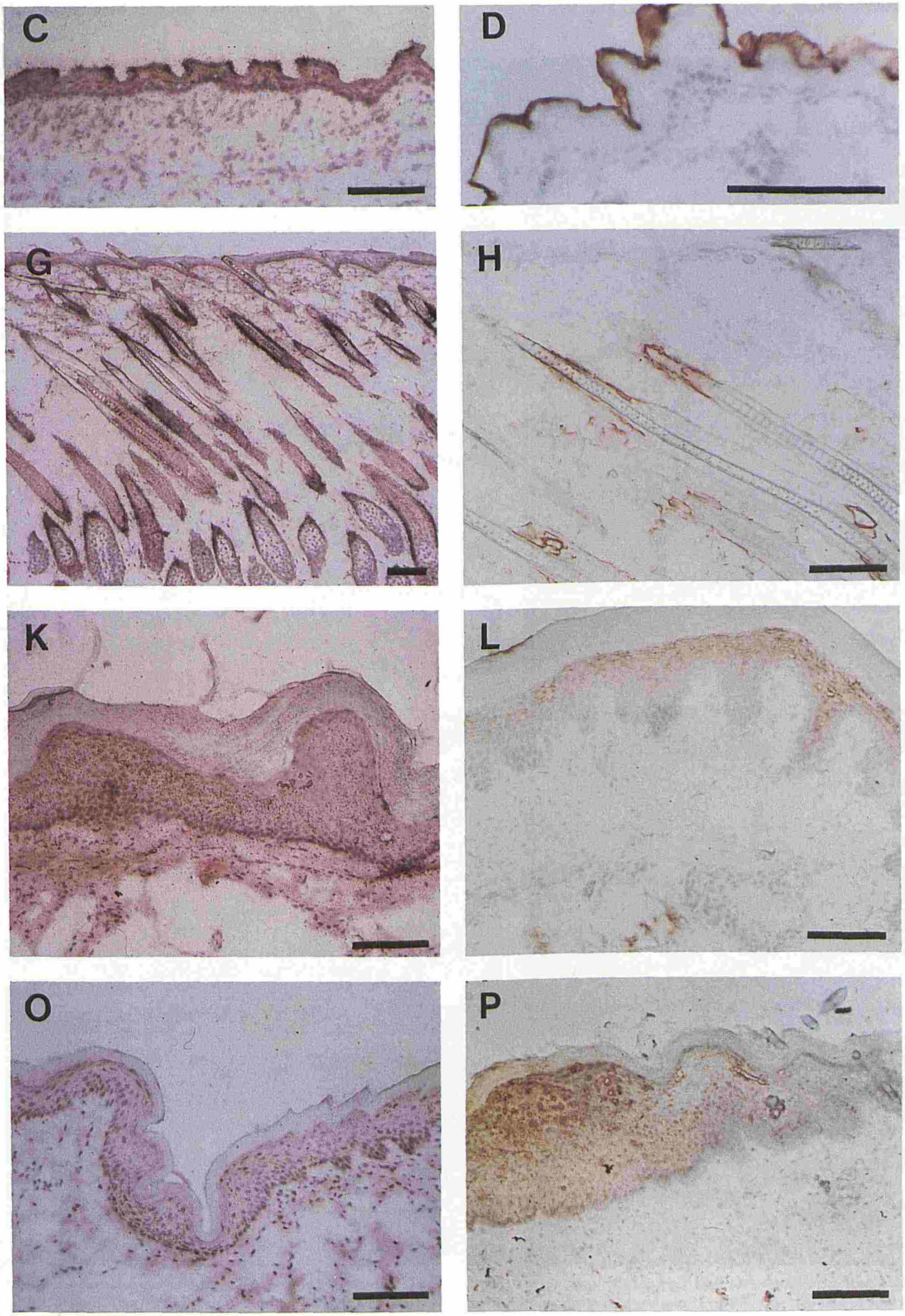


Figure 6. SPR1 is expressed in specific and restricted cell types of external epithelia as revealed by *in situ* hybridization and immunohistochemical staining. Tissues were from: 17-d-old fetus (A–D); 9-d-old mouse (E–H); foot pad of adult mouse (I–L); and upper lip of adult mouse (M–P). *In situ* hybridization was performed using ^{35}S-labeled SPR1 RNA, using a sense (darkfield images [*A,E,I,M*]; bright filed images [*C,G,K,O*])



or anti-sense (darkfield images [*B,F,J,N*]) probe. Frozen sections were analyzed for the presence of SPR1 protein using the affinity-purified SPR1-Cterm antibody (*D,H,L,P*). Antibody binding was visualized by horseradish peroxidase-conjugated second antibody. Note that the sense probe yields nonspecific labeling of the hair medulla (*E*) and cornified layers of footpad epidermis (*I*). *Scale bars*, 100 μm.

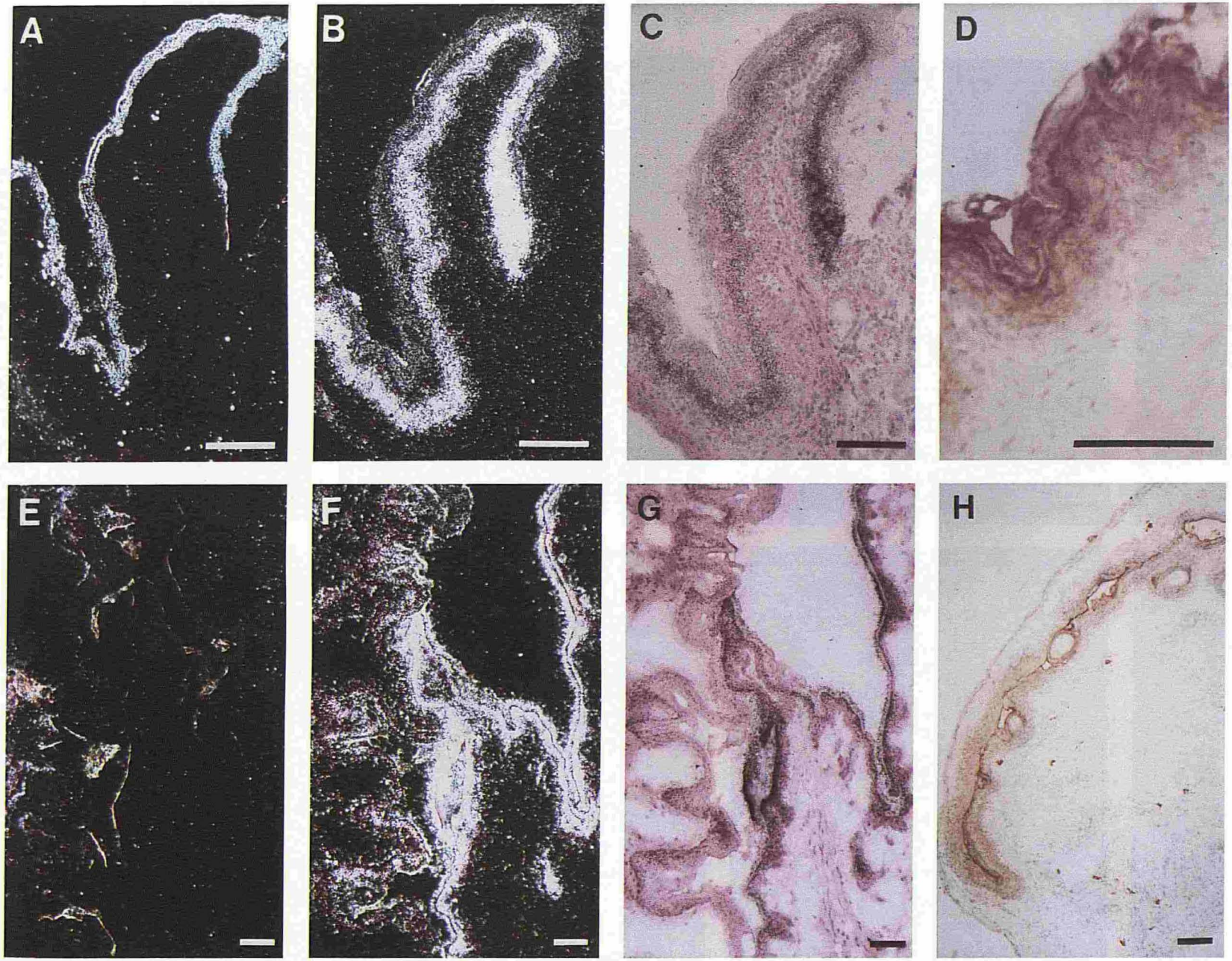
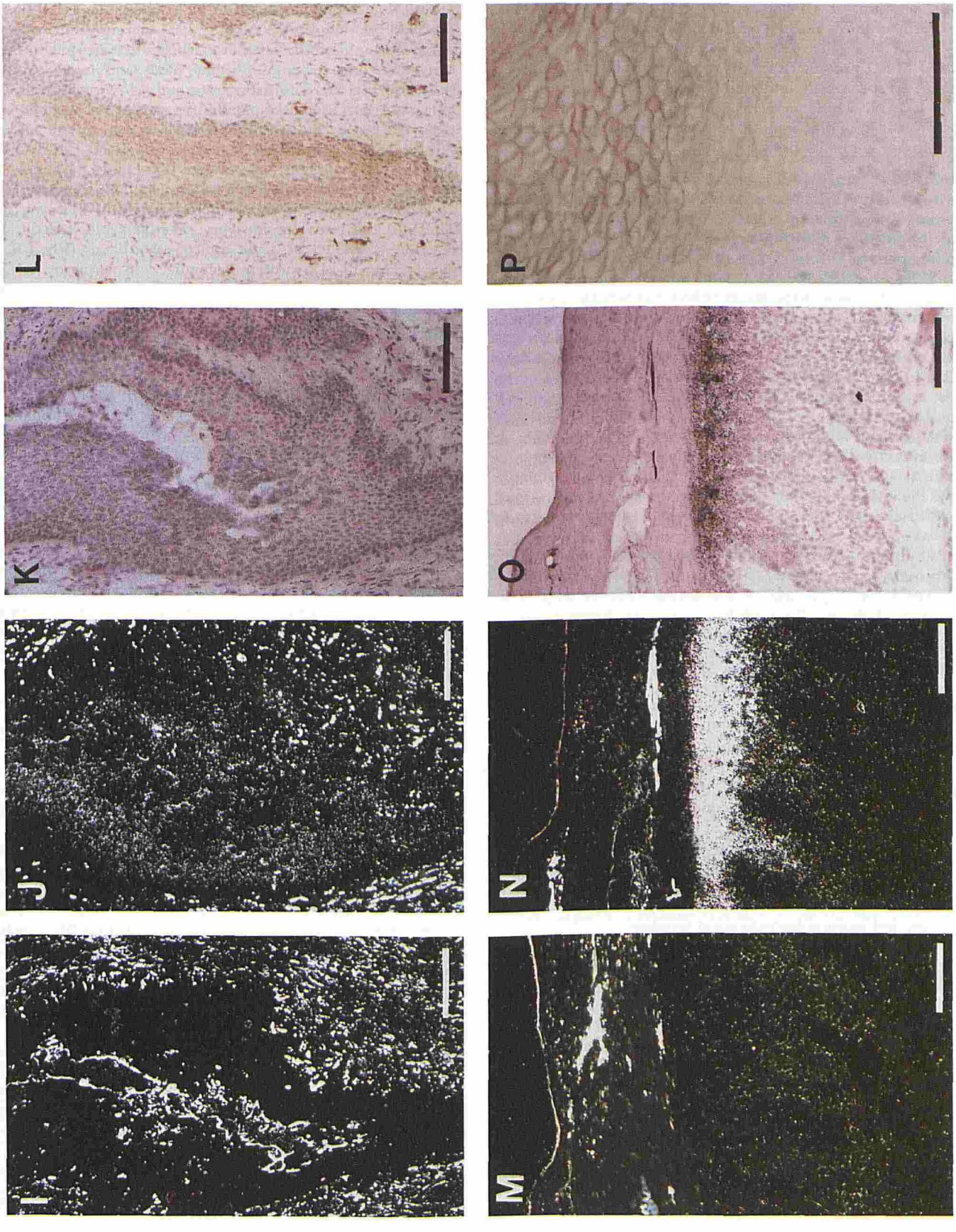


Figure 7. SPR1 is expressed in specific and restricted cell types of internal epithelia as well as mouse papillomas as revealed by *in situ* hybridization and immunohistochemical staining. The tissues used are: forestomach (*A–D*); penis (E–H); vagina (I–L); and papilloma (M–P). *In situ* hybridization was performed using ^{35}S-labeled SPR1 RNA, using a sense (dark filed images [*A,E,I,M*]; bright field images [*C,G,K,O*]) or antisense (dark filed images [*B,F,J,N*]) probe. Frozen sections were analyzed for the presence of SPR1 protein using the affinity-purified SPR1-Cterm antibody (*D,H,L,P*). Antibody binding was visualized by horseradish peroxidase conjugated second antibody. Note that the sense probe nonspecifically decorates the cornified layers of the forestomach (*A*) and vagina (*I*). *Scale bars*, 100 μm.



In Vitro* Synthesized SPR1 Protein Is Recognized by an Antibody Generated Against the C-Terminal Peptide** The mouse SPR1 protein was initially characterized *in vitro*. To determine the apparent molecular weight of SPR1 protein on SDS-PAGE, the SPR1a protein was synthesized *in vitro* in reticulocyte lysates using T7-generated sense SPR1a RNA as a template (**Fig 3*A). The calculated molecular weight for SPR1a protein is 15.8 kDa, but *in vitro* synthesized SPR1a migrated abnormally as an approximately 30 kDa protein (**Fig 3*A***, *lane 1*). The reason for this anomalous migration is unclear, but might be due to the exceptionally high content of prolines, as was suggested previously [22]. Control translation of anti-sense RNA template produced no

significant amount of labeled protein (**Fig 3*A***, *lane 2*). The SPR1a-Cterm antibody reacted with the *in vitro* synthesized SPR1a on a Western blot (**Fig 3*B***, *lane 1*), but not with the *in vitro* synthesized SPR2 protein (Kartasova *et al*, unpublished observations), and was specifically inhibited by the SPR1a-Cterm peptide (**Fig 3*B***, *lane 2*). These *in vitro* expression data were used to interpret the Western blot analyses of the SPR1 protein expression *in vivo* in mouse epithelial tissues.

SPR1 mRNA Is Expressed in Fetal Periderm, Hair Follicles of Newborn and Adult Mouse Skin, and in Benign Mouse Papilloma Expression of SPR1 in mouse skin was initially analyzed by Northern blotting using a labeled polymerase chain reaction fragment of SPR1a cDNA as a probe. Since the homology between SPR1a and SPR1b is 85% in the coding region, the SPR1a probe does not discriminate between SPR1a and SPR1b sequences. As shown in **Fig 4**, expression of SPR1 mRNA in mouse fetal skin was upregulated around day 16 and persisted in the newborn and adult skin that includes epidermal appendages. In the case of the fetal skin, however, the signal is most likely derived from the periderm, as indicated by *in situ* hybridization and by immunohistochemical analysis for the SPR1 (see below). In newborn and adult skin, the detected SPR1 mRNA expression might be entirely due to the SPR1 mRNA expression in hair follicles, rather than in the interfollicular epidermis, since SPR1 was detected in hair follicles, and not in the interfollicular epidermis, both by *in situ* hybridization and by SPR1-specific antibody (see below). No mRNA expression of SPR1 was found by Northern blotting in other mouse tissues tested (i.e., brain, heart, intestine, kidney, liver, lung, ovary, skeletal muscle, spleen, testis, or thymus [not shown]). SPR1 mRNA expression in prenatal and postnatal skin, as well as in various epithelial tissues of the adult mouse, was analyzed further by *in situ* hybridization (see below).

SPR1a Protein Is Expressed in a Wide Variety of Internal and External Keratinizing Epithelia The analysis of the expression of the SPR1 proteins by Western blotting is complicated because much of the monomeric SPR1 proteins may be cross-linked to larger structures. For example, most of the loricrin in the epidermis is cross-linked [7,29], and only traces (~0.1%) can be recovered in monomeric form [7]. In addition, the available sequencing data on cross-linked peptides from human foreskin epidermal CEs indicates that mouse SPR1 is probably also cross-linked by transglutaminases and, moreover, in sequences that provide specificity for the production of the antibody [44]. Thus, the epitopes in some cross-linked forms are likely to be masked. The data are therefore presented in parallel with the *in situ* hybridization and immunohistochemical staining.

While the Northern and *in situ* hybridization data probably do not discriminate the expression of SPR1a and SPR1b mRNA because of high sequence homology **(Fig 4)**, it is not known whether the SPR1a-Cterm antibody will cross-react with the SPR1b protein, although there is significant sequence identity in their terminal sequences **(Fig 1)**. Thus, the absence of bands of 16–18 kDa expected for the SPR1b protein in papilloma extracts **(Fig 5*B*)** could be because either the SPR1a-Cterm antibody does not cross-react with the SPR1b protein, or the SPR1b protein is in fact not synthesized.

To study the regulation of SPR1a expression in developing mouse fetal epidermis, fetal skins from day 15 to 18 **(Fig 5*A*)** were excised and total protein was analyzed for the presence of SPR1a. No SPR1a staining was visible on day 15. On day 16 and 17, one or two major bands of 30 and 32 kDa, and several high–molecular weight bands were present. On day 18, the 30- and 32-kDa proteins were no longer detectable, but weak higher molecular weight bands remained visible. Control staining of the same blot for keratin 6 which is expressed in periderm but not in epidermis of the fetus (Dr. S. H. Yuspa, NCI, Bethesda, personal communication), detected positive keratin 6 staining in samples from day 16 to 18 **(Fig 5*A*)**. The SPR1a expression was analyzed further in trunk skin of mice at birth and 3 d after birth, and in adult foot pad. There was almost no SPR1 detectable in newborn skin. In skin from a 3-d-old mouse, one prominent band of 30 kDa was detected. In the adult foot pad two bands of 30 and 32 kDa were present.

Expression patterns in other epithelial tissues, such as the esophagus, forestomach, tongue, vagina, penis, and in skin papilloma are shown in **Fig 5*B***. The strongest signal, as two bands of 30 and 32 kDa, was detected in the papilloma sample. (A band of 16–18 kDa expected for the SPR1b protein was not seen by the antibody). In the tongue and forestomach, two bands of 30 and 50 kDa were detected. In the vagina, there were two bands, 30 and 32 kDa. In the penis, one band of 30 kDa was detected. In the esophagus, only a weakly reacting protein of approximately 65 kDa was detected. The SPR1a-Cterm peptide very effectively blocked the antibody reaction with 30-, 32-, 50-, and 65-kDa bands **(Fig 5*B***, *last two lanes*). The 30-kDa protein recognized by antibody on Western blots is probably mouse SPR1a protein since it has the same mobility as the *in vitro* synthesized SPR1a protein. Other bands detected on Western blots might be post-translational modifications of SPR1a (32-kDa protein), related members of the SPR1 or SPR3 subfamilies (50 kDa protein), or SPR1a cross-linked to itself or to other proteins (multiple high–molecular weight bands in samples from periderm). Since human SPR1 and SPR3 are highly homologous at the C terminus [13], the SPR1a-Cterm antibody might also cross-react with the mouse SPR3 protein.

***In Situ* Hybridization Reveals Specific Cell Types That Synthesize SPR1 mRNA in Various Epithelia, Including Tissues Found Positive by Northern Blotting** By *in situ* hybridization, expression of SPR1 mRNA was detected in periderm of the fetal skin **(Fig 6*B,C*)**, in hair follicles of newborn (not shown) and 9-d-old mouse **(Fig 6*F,G*)**, and in the thickened area of the foot pad **(Fig 6*J,K*)**, and lips **(Fig 6*N,O*)**. In the foot pad, the signal was found in suprabasal cell layers **(Fig 6*K*)**. No signal was detected in interfollicular epidermis **(Fig 6*F,G*)**. In contrast to epidermis, a high level of SPR1 mRNA expression was found in epithelia from the forestomach **(Fig 7*B,C*)** and penis **(Fig 7*F,G*)**, and in some areas of benign squamous papilloma **(Fig 7*N,O*)**. Other epithelia expressing SPR1 mRNA include the vagina **(Fig 7*J,K*)**, tongue, and esophagus (not shown, but see [12]).

Immunohistochemical Staining for SPR1a Protein Correlates with the Expression of mRNA as Seen by *In Situ* Hybridization The SPR1a-Cterm antibody was used for immunohistochemical analysis of different mouse tissues. As for Western blot analysis, the ability to detect SPR1 in tissues by immunostaining may be limited by epitope masking due to crosslinking [44]. During fetal development, strong SPR1a staining was detected in the periderm of 16- and 17-d-old fetuses **(Fig 6*D*)**, which correlated closely with keratin 6 staining in the periderm (data not shown). No visible SPR1a staining was detected in the developing epidermis. In the newborn mouse, SPR1a protein expression in the interfollicular epidermis was absent, and it was not detectable in mouse interfollicular epidermis from day 1 to 19 **(Fig 6*H*,** 9 d after birth), or in adult mice. In contrast, SPR1a staining was visible in differentiating epidermal layers of the upper and lower lips **(Fig 6*P*,** transition between lip and skin is shown), and in differentiating layers of the foot pad, but not in cornified cells **(Fig 6*L*)**. Staining was also prominent in the restricted regions of the inner root sheath of hair follicles **(Fig 6*H*)**. The presence of SPR1 in hair follicles explains the positive signal in Northern **(Fig 4)** and Western **(Fig 5*A*)** blots since these samples of skin contain anagen hair follicles. In other epithelia, positive staining was observed in the differentiating epidermal layers of the keratinizing forestomach (very strong staining, **Fig 7*D***, but not in the simple epithelium of the glandular stomach), in the stratified squamous epithelium of the vagina (lightly stained, **Fig 7*L***), and in the skin of the penis (strong staining at the base of the penis is shown in **Fig 7*H***), in the tongue, and in the esophagus (not shown). In mouse papillomas, SPR1a expression was detected in both the differentiating layers and the cornified layers **(Fig 7*P*)** with peripheral staining in the cornified cells. In all cases, antibody reaction was completely inhibited by the SPR1a-Cterm peptide in all tested tissues (not shown).

DISCUSSION

In the present study, mouse SPR1 cDNA clones were isolated and used to explore the expression of the mouse SPR1 gene at the RNA and protein levels. Two different SPR1 sequences, SPR1a and SPR1b, were identified in a mouse papilloma cDNA library. Sequence analyses suggested that SPR1b contains a deletion in the central repeated domain relative to SPR1a. Remarkably, this deletion is a codon deletion preserving the reading frame. Similar multiple codon deletions were observed in the central repeat of the involucrin genes of different mouse strains and of rat [9]. Whether the deletion in SPR1b is also present in corresponding SPR gene in the mouse genome remains to be established.

Comparison between SPR1 proteins from several species suggest the importance of the length and amino acid sequence of the N and C termini of the SPR1 proteins, which no doubt reflects the important functional significance of these glutamine- and lysine-rich sequences in cross-linking by transglutaminases [44]. Similarly, the N and C termini are conserved in loricrins [20,29], which are likewise critically involved in cross-linking reactions with SPRs [44]. The variation in the number of repeats, from six repeats in human and monkey to 13 repeats in mouse SPR1a, suggests that the number of repeats is not stringent. Similar observations have been made in profilaggrin genes: while the human profilaggrin gene has 10–12 filaggrin repeats [28], the mouse gene has >20 repeats, and the number varies further with the strain of the mouse [37].

Using anti-sense SPR1a riboprobes and an SPR1a-Cterm antibody, SPR1a expression was studied in normal mouse epithelial tissues during development, at birth, and in adult animals, as well as in mouse papillomas by *in situ* hybridization and by immunohistochemistry. In fetal skin, SPR1a expression is localized to the periderm rather than to the developing keratinizing epidermis. A similar observation was made previously in human fetal epidermis [23]. The first positive SPR immunostaining was detected on day 16, although some weak staining was present on day 15. By day 18, immunostaining becomes patchy as periderm begins to slough. Expression of SPR1 mRNA and protein followed the same time course of induction. During fetal development, epidermis consists initially of basal and periderm cell layers [5,51]. Both are formed from the embryonic ectoderm around day 12. The basal layer subsequently gives rise to the epidermis. Periderm is mitotically active until day 14, but then terminally differentiates, and finally, is shed around day 18–19, when the epidermis has stratified into several layers [51]. Thus, expression of SPR1a protein correlates well with the cessation of proliferation and induction of terminal differentiation in the periderm, in concordance with keratin 6 expression in the periderm. This indicates that the periderm CE may share some of the precursors seen in the epidermal CE (involucrin [50] and SPRs, but not loricrin [5,6,57]) and thus may be initially assembled and organized in a similar way.

Recent analyses of the amino acid compositions of newborn mouse epidermal CEs indicated that SPRs might comprise up to 5% of the mature CEs [43,45]. However, in this study, no SPR1a expression was detected in fetal, newborn, and adult interfollicular trunk mouse epidermis by *in situ* hybridization and immunohistochemistry. It is possible that the amount of SPR1a protein in epidermis is below the detection level. A more likely possibility is that other proteins with a similar amino acid composition, such as SPR2, or an as yet unidentified CE protein, may participate in CE assembly in mouse epidermis rather than SPR1a. This issue will be clarified by direct sequencing of the cross-linked peptides from mouse CEs. Likewise, SPR1a expression is low in normal interfollicular human epidermis although more abundant in foreskin epidermis [21]. Based on direct sequencing data, it represents about 2% of human foreskin epidermal CEs [44].

In the adult mouse, SPR1 mRNA and SPR1a protein were abundantly expressed in the differentiating layers of thickened tissues such as the epidermis of the foot pad and lips, and tongue epithelium. These observations suggest that SPR1 expression may correlate with epithelial and cornified layer thickness. In most cases, the immunostaining diminished greatly in the cornified layers. This observation is similar to involucrin [9] and loricrin [29] staining in mouse skin, and is likely to be the result of loss of antibody epitope due to specific transglutaminase cross-linking [44]. In contrast, in the forestomach, which is a stratified squamous keratinizing epithelium in mouse, prominent cell peripheral immunostaining was seen in the cornified layers. In this case, the SPR1a-Cterm antibody might be reacting with an epitope present on two related SPR proteins, as judged by Western blotting **(Fig 5*B*)**. Cell peripheral SPR1a immunostaining of the cornified layers was also seen in benign papilloma. The latter pattern of expression resembles loricrin [29]. This may mean that in these tissues SPR proteins are less cross-linked, or cross-linking involves primarily other portions of the molecule, rather than a cross-bridging function through terminal sequences [44].

A further common characteristic of several SPR1a positive squamous epithelia is the association with tissues for which extreme flexibility is required, (i.e., the penis, vagina, lips, tongue, esophagus, forestomach, foot pad, and periderm). The increased expression of SPR1a in these epithelia might lead to an increase in the ratio of the SPR1a to other proteins in these CEs and thus change the mechanical properties of the CEs. Likewise, the abundant expression of SPR1a in mouse hair follicles may be consistent with this idea, but the possibility that SPR1a might perform another so far undefined role in the differentiation of the inner root sheath cells must be explored. In corraboration with these ideas, recently we have made computer-based predictions of the secondary structures of the central repeat of the SPR proteins.[1] The adjacent prolines and charged residues of the central repeating motifs are likely to form a folded structure with one or more β-turns to form a rather rigid structure. From the nature of these repeats, it was concluded that the repeats of SPR2 are more rigid than those of SPR1 proteins, which in turn are more rigid than those of the human SPR3 protein. Thus, the mechanical attributes of the CE and epithelia as a whole could vary depending on the type and amount of the SPR expressed in the tissue, and thereby render the tissue uniquely adapted to function.

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Note added in proof: We have completed sequencing of the mouse genomic SPR1a and SPR1b by PCR using specific primers and genomic DNA from three different mouse strains: CD-1, Balb/c and C57black. The deletion of one codon in the third repeat of SPR1a was confirmed. The SPR1b gene appeared to contain 14 central repeats in all the strains, instead of 7 repeats reported in Figures 1 and 2. Thus, the deletion in SPR1b might be due to the cDNA cloning artefact. The calculated molecular weight of SPR1b protein is 16,636. Therefore, SPR1b protein is predicted to migrate slower than SPR1a protein on SDS-PAGE, and it might be the 32kDa protein in Figure 5. The full-length SPR1b sequence is available in the Genebank under the accession number X91825.

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